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An Enzymically Synthesized RNA of Alternating Base Sequence: Physical and Chemical Characterization.

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The reaction catalysed by the RNA polymerase from *Escherichia coli* has been studied using dAT† copolymer as primer. The product of the reaction, rAU copolymer, contains a regularly alternating sequence of AMP and UMP residues. Measurements of sedimentation velocity, viscosity, optical rotation and optical density melting indicate that rAU can exist as a rigid, rod-like helix which is qualitatively similar to the dAT helix. However, the rAU helix shows a greater stability to thermal denaturation, a higher hyperchromic effect on denaturation, and a higher optical rotation. Attempts to form a hybrid molecule between rAU and dA$\overline{\text{BU}}$, a dAT analogue, were unsuccessful. Furthermore no evidence of such a hybrid as a stable intermediate in the enzymic reaction could be obtained.

1. Introduction

The DNA-directed enzymic synthesis of RNA from the ribonucleoside triphosphates has been studied in a number of laboratories (Weiss & Nakamoto, 1962*a*,*b*; Furth, Hurwitz & Anders, 1962; Hurwitz, Furth, Anders & Evans, 1962; Chamberlin & Berg, 1962; Stevens, 1960, 1961). To study the mechanism of the reaction and the properties of the newly synthesized RNA, we have examined the dAT-directed formation of rAU using *E. coli* RNA polymerase. The advantages of dAT as primer are: (1) its primary structure is specified exactly (Schachman, Adler, Radding, Lehman & Kornberg, 1960); (2) it has the helical secondary structure found in natural DNA (Davies & Baldwin, 1963), and its behavior in solution has already been the subject of physical–chemical investigation (Schachman *et al.*, 1960; Inman & Baldwin, 1962*a*,*b*); (3) some information as to the mechanism of the dAT-primed synthesis of dAT with *E. coli* DNA polymerase (Wake & Baldwin, 1962) is available; and (4) dAT is a very effective primer for polyribonucleotide synthesis (the amount of rAU produced in the reaction can exceed by 50 times the amount of dAT added as primer).

In agreement with the earlier findings of Furth, Hurwitz & Goldman (1961), our studies indicate that rAU contains a regularly alternating sequence of AMP and UMP residues. Furthermore, measurements of sedimentation velocity, viscosity, optical

† Abbreviations: dAT, deoxyadenylate-deoxythymidylate co-polymer; dA$\overline{\text{BU}}$, deoxyadenylate-deoxybromouridylate copolymer; dAU, deoxyadenylate-deoxyuridylate copolymer; rAU, riboadenylate-ribouridylate copolymer; and rA:rU, the helical complex of polyriboadenylic acid with polyribouridylic acid. The nomenclature used is that set forth by Inman and Baldwin (1962*a*). AMP and ATP are used for the adenosine-5′ mono- and triphosphates, respectively; a similar notation is used for the uridine (U) derivatives and their deoxy analogues, thymine (dT), deoxyuridine (dU) and deoxy-5′-bromouridine (d$\overline{\text{BU}}$). P_i is used for inorganic phosphate, PP_i for inorganic pyrophosphate. ϵ_P^{260} is the molar extinction coefficient at 260 mμ relative to phosphorus.

rotation and optical density melting indicate that rAU can exist as a rigid, rod-like helix which is qualitatively similar to the parent dAT helix. The rAU helix differs from the analogous dAT helix in its greater stability to thermal denaturation, its higher hyperchromic effect on denaturation and its higher optical rotation. There are also differences in the X-ray diffraction patterns obtained from the two species.

In a mixture of rAU and dAT isolated from the enzymic reaction, or prepared by combining isolated rAU and dAT, one finds two optical density transitions at the melting zones of dAT and rAU; there is no evidence for a component of intermediate thermal stability. Moreover, these mixtures give discrete bands in a cesium sulfate density gradient with no indication of a band of intermediate density. These data indicate that if a hybrid molecule between the dAT primer and rAU product is formed during the enzymic reaction, it has only a transient existence, in agreement with the earlier findings of Geiduschek, Nakamoto & Weiss (1961). Although hybrids of dAT and dA$\overline{\text{BU}}$ have been made previously (Inman & Baldwin, 1962*b*), attempts to form such a hybrid physically between rAU and dA$\overline{\text{BU}}$, the bromouracil-containing analogue of dAT, were not successful.

2. Materials and Methods

Tricyclohexylammonium phospho-enol pyruvate sodium ATP, and crystalline rabbit muscle pyruvate kinase were obtained from Sigma Chemical Company, St. Louis, Missouri. [8-^{14}C]ATP and sodium UTP were purchased from Schwartz BioResearch, Inc., Orangeburg, New York. [^{14}C]UTP was prepared as previously reported (Chamberlin & Berg, 1962).

Phenol was Mallinckrodt Analytical Reagent grade liquefied phenol, containing no preservatives, and was used without further purification. Sodium lauryl sulfate was obtained from the Fischer Scientific Company, St. Louis, Missouri, and was recrystallized twice from ethanol before use. Cesium sulfate was purified as before (Wake & Baldwin, 1962) and then recrystallized from 0·01M M-EDTA. Amberlite XE-64 was prepared according to Hirs, Moore & Stein (1953) and was equilibrated with 0·05 M-sodium citrate buffer, pH 5·4.

dAT copolymer was prepared according to Schachman *et al.* (1960); dAU copolymer was prepared with dUTP (Bessman *et al.*, 1958) by Mr. E. Elson using a similar method. [β, γ-^{32}P]ATP was prepared as previously described (Preiss, Dieckmann & Berg, 1961); [α-^{32}P]ATP was obtained by enzymic phosphorylation of [^{32}P]AMP (Lehman, Bessman, Simms & Kornberg, 1958) prepared using the method of Straus & Goldwasser (1961) from isopropylidene adenosine purchased from California Corporation for Biochemical Research, Los Angeles, California. $\overline{\text{BU}}$TP was prepared by direct bromination of UTP in formamide solution, and purified by chromatography on Dowex-1 chloride (Chamberlin & Berg, to be published). [α-^{32}P]UTP was prepared from [α-^{32}P]CTP (Chamberlin & Berg, 1962) by deamination with nitrous acid (Bessman *et al.*, 1958). RNA polymerase was Fraction 4 enzyme prepared as previously described (Chamberlin & Berg, 1962). The pH of buffer solutions was determined at 0·05 M concentration unless stated otherwise. Except where otherwise noted, sodium citrate buffer contained 2 parts sodium chloride to 1 part trisodium citrate on a molar basis, and had a pH of about 7·5. Concentrations are expressed in terms of the total sodium ion concentration.

Inorganic phosphorus was determined by the method of Chen, Toribara & Warner (1956) as modified by Ames & Dubin (1960). Total nucleic acid P was determined after ashing the sample for 1 min with 0·06 ml. of 10% $Mg(NO_3)_2$ in ethanol. The precision of the determination was estimated to be $\pm 5\%$. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Methods used for viscometry, optical density melting and density gradient sedimentation determinations were as previously reported (Inman & Baldwin, 1962*a*,*b*; Wake & Baldwin, 1962). Viscometry determinations were carried out in a capillary viscometer with a flow time of 180 sec for water at 20°C. Beckman fused silica cuvettes fitted with ground glass stoppers were used for measuring spectra and optical density melting. They were washed in a solution of

1·5 N-HCl in 50% ethanol, rinsed in distilled water and sterilized in a 180°C oven for 1 hr before use with high molecular weight RNA preparations. Evaporation during melting determinations was prevented by covering the solution with a layer of washed mineral oil as previously described (Inman & Baldwin, 1962*b*). Optical rotatory dispersion measurements were carried out in a Rudolph photoelectric polarimeter, using a high-pressure mercury lamp as source. The cell was 100 mm in length and 3 mm in diameter; it was prepared for use in the same manner as the cuvettes.

Large scale synthesis of rAU copolymer

The reaction mixture contained in a final volume of 39 ml.: 1·4 m-moles of tris, pH 8·0, 40 μmoles of $MnCl_2$, 200 μmoles of $MgCl_2$, 430 μmoles of 2-mercaptoethanol, 45 μmoles of ATP, 45 μmoles of UTP, 100 μg of pyruvate kinase, 30 μmoles of PEP, 400 μmoles of KCl, 1·1 μmoles of dAT, and 9·2 mg of Fraction 4 RNA polymerase. After incubation at 37°C for 2 hr, 3·5 ml. of 5% sodium lauryl sulfate were added and the mixture was incubated for 3 min at 37°C, then chilled at 0°C for 2 hr. The resulting precipitate was removed by centrifugation, and 1·9 ml. of 1 M-sodium citrate, pH 5·4, were added. The mixture was warmed to 25°C and extracted three times with an equal volume of phenol. The pooled phenol layers were then extracted with two 15 ml. portions of 0·05 M-sodium citrate, pH 5·4. The pooled aqueous layers were filtered through an Amberlite XE-64 column as described below, and the rAU was precipitated from the effluent by the addition of NaCl solution to 0·5 M and two volumes of cold ethanol. The precipitate was dissolved in 10 ml. of 0·05 M-sodium citrate, pH 5·4, and dialysed for 48 hr against two changes of 500 ml. each of 0·5 M-sodium citrate–0·01 M-EDTA, pH 7·5, then dialysed for 48 hr against two changes of 500 ml. each of 0·01 M-EDTA, pH 7·5, then for 48 hr against two changes of 500 ml. each of 0·5 M-sodium citrate, and finally against 500 ml. of 0·05 M-sodium citrate–0·001 M-EDTA for 12 hr. The resulting preparation, containing 52 μmoles of rAU copolymer, was stored at −15°C. Nucleic acid concentrations are expressed here and elsewhere as μmoles of nucleotide. The protein content of rAU samples prepared in this manner was less than 0·5% as determined by the Lowry method. The amount of dAT primer remaining in the purified rAU (a maximum contamination of 2%) was not determined.

rA$\overline{\text{BU}}$ was prepared in a similar manner from ATP and $\overline{\text{BU}}$TP.

Handling of rAU copolymer preparations

Early preparations of rAU copolymer were unstable, becoming non-sedimentable and acid-soluble when heated to 70°C or when stored for several months at −15°C. A similar instability of other high molecular weight RNA preparations has been noted previously (Fraenkel-Conrat & Singer, 1958; Fraenkel-Conrat, Singer & Tsugita, 1958; Aronson & McCarthy, 1961; Holley, Apgar & Merrill, 1961). The degradation may be due to RNase present in the enzyme preparation, introduced by handling (Holley *et al.*, 1961), or released by trace bacterial contaminants. The following precautions were therefore taken during the isolation and use of rAU preparations.

(1) Sterile glassware, reagents and techniques were used in all experiments in which the physical integrity of the polymer was required.

(2) Dialysis tubing was handled with sterile gloves; it was prepared by heating to 80°C for 30 min in 0·01 M-EDTA, pH 7, soaked overnight in fresh 0·01 M-EDTA, pH 7, and stored in 0·001 M-EDTA, pH 7.

(3) Polymer preparations which could not be sterilized—such as the dAT copolymer used to prime for rAU synthesis—were passed through a 5 cm × 1 cm^2 column of Amberlite XE-64 in 0·05 M-sodium citrate, pH 5·4.

(4) Sterile, quartz distilled water was used.

The stability of rAU preparations obtained and handled using these techniques was investigated by determining the effect of heating at 70°C in 0·05 M-sodium citrate, pH 7·5, on the mean sedimentation coefficient in 0·05 M-sodium citrate buffer at 25°C. The unheated preparation had a sedimentation coefficient of 8·5$\pm$0·3 s; heating for 15 min reduced this to 8·1 s, and after 6 hr the sedimentation coefficient had fallen to 7·2. The sedimentation coefficient of this same unheated preparation decreased to 7·8 s on storage for 5 months at −15°C, the preparation having been frozen and thawed, and samples withdrawn frequently during this period.

3. Results

(a) *Characterization of the products of the dAT-primed reaction*

(i) *Stoichiometry*

The stoichiometry of the dAT-primed reaction was determined using [^{14}C]UTP and [β, γ-^{32}P]ATP. The results (Table 1) show that the amounts of ATP and UTP consumed in the reaction were equal, and that the amount of PP_i released was equivalent to the quantity of nucleotide incorporated into rAU. As expected, the specific activity of the PP_i produced was half that of the ATP added, due to dilution by an equivalent amount of unlabeled PP_i released from UTP.

TABLE 1

Stoichiometry of the dAT-primed synthesis of rAU

Component	Initial		Final		Δ
	Amount	Specific activity	Amount	Specific activity	
	mμmoles	cts/min/mμmole	mμmoles	cts/min/mμmole	mμmoles
rAU copolymer	0	—	552	—	+552
P_i	0	—	59	—	+ 59
PP_i	0	—	480	204	+480
ATP	512	407	234	417	−278
UTP	487	124	214	134	−273

The reaction mixture contained in 0·51 ml.: 20 μmoles of tris, pH 7·9; 0·5 μmole of $MnCl_2$; 2 μmoles of $MgCl_2$; 6 μmoles of 2-mercaptoethanol; 512 mμmoles of [β, γ-^{32}P]ATP; 487 mμmoles of [^{14}C]UTP; 100 mμmoles of dAT copolymer; and 48 μg of Fraction 4 RNA polymerase.

After 30 min at 37°C, the mixture was chilled and 1·2 mg of serum albumin and 1·3 ml. of 3·5% trichloroacetic acid (TCA) were added. The precipitate was washed 4 times with 1·0 ml. portions of TCA by centrifugation and resuspension. The precipitate was then dissolved in ammonia, dried on a planchet, and counted to determine the amount of rAU synthesized. The combined TCA supernatant fluids were extracted 3 times with 5 ml. portions of cold ether. Air was bubbled through the solution for 15 min to remove the ether, and 0·2 ml. 2N-ammonia was added to give a pH > 8·5. The mixture was diluted to 25 ml. and placed on a 5 cm × 1 cm^2 column of Dowex 1-Cl (10% cross-linking). P_i and PP_i were eluted separately in that order with 0·01 N-HCL–0·05 N-KCl, ATP was removed with 0·01 N-HCl–0·10 N-KCl, and then UTP was eluted with 0·1 N-HCl–0·2 N-KCl.

The products were identified by their position on the chromatogram and as follows: P by reaction in the Chen phosphate determination, PP_i by its failure to react fully in the latter assay unless treated with 0·5 N-HCl for 15 min at 100°C or with crystalline yeast pyrophosphatase (Heppel, 1955), and ATP and UTP by their spectral properties. The specific activities of the ATP, UTP and PP_i were constant in individual fractions of each chromatographic peak.

(ii) *Nearest neighbor studies*

The alternating sequence of the nucleotides in the rAU product was established by ^{32}P transfer studies, using as substrates alternately [α-^{32}P]ATP and [α-^{32}P]UTP, and measuring the distribution of ^{32}P in the alkaline hydrolysis products (Heidelberger, Harbers, Leibman, Takagi & Potter, 1956). As shown in Table 2, the radioactivity incorporated as [^{32}P]-5′-AMP appears only in 2′-(3′)UMP. Label incorporated as [^{32}P]-5′-UMP is transferred solely to 2′-(3′)AMP. Thus even when over 30 times more rAU is made than dAT primer added, the alternating sequence of the primer is faithfully preserved in the product.

TABLE 2

^{32}P-Transfer from [α-^{32}P]-ATP and [α-^{32}P]-UTP in enzymically synthesized rAU copolymer

Substrates	Radioactivity in isolated mononucleotides	
	2′-(3′)AMP	2′-(3′)UMP
	cts/min	
[α-^{32}P]ATP + UTP	2	9381
ATP + [α-^{32}P]UTP	2593	0

The reaction mixture contained in 0·27 ml.: 1 μmole of tris, pH 7·9; 0·25 μmole of $MnCl_2$; 1·0 μmole of $MgCl_2$; 3 μmoles of 2-mercaptoethanol; 8·5 mμmoles of dAT; 300 mμmoles of [α-^{32}P]ATP ($1{\cdot}7 \times 10^6$ cts/min/μmole); 300 mμmoles of UTP; and 83 μg of Fraction 4 enzyme. In the second experiment, the concentrations were identical except that [α-^{32}P]UTP ($3{\cdot}6 \times 10^5$ cts/min/μmole) was the labeled precursor.

The reaction mixture was incubated for 90 min at 37°C during which time approximately 260 mμmoles of rAU were synthesized in each reaction. The reaction was stopped by the addition of 1·2 mg of serum albumin and 3·5 ml. cold 3·5% perchloric acid (PCA); the precipitate was washed 4 times by centrifugation and resuspension in cold PCA. The precipitate was then suspended in 0·1 ml. H_2O, neutralized with KOH, and made up to 0·3 M in KOH. After 18 hr at 37°C, the reaction mixture was acidified to pH 4 with PCA and the precipitate removed by centrifugation. A portion was mixed with 100 mμmoles each of 2′-(3′)AMP and 2′-(3′)UMP and the nucleotides separated by electrophoresis (Markham & Smith, 1952). The spots corresponding to AMP and UMP were located with a u.v. lamp, cut out, and eluted with 0·01 M-HCl for 8 hr, then portions were dried on planchets and the radioactivity of each component determined. Recovery of the counts on electrophoresis was greater than 95% in each case.

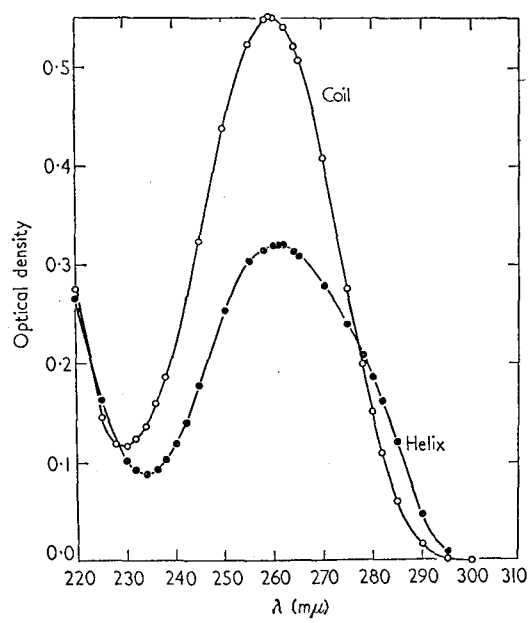

FIG. 1. *Spectrum of rAU copolymer in helix and coil forms.* Data were taken in 0·01 M-sodium citrate buffer at a nucleic acid concentration of 53 mμmoles/ml. Data for the helix spectrum were obtained at 25°C, those for the coil at 56°C.

(iii) *Spectra*

The spectra of the helix and coil forms of rAU are shown in Fig. 1. For the helix a value of ϵ_P^{260} of 6100 was obtained ($\lambda_{max} = 261$ mμ), for the coil $\epsilon_P^{260} = 10{,}400$ ($\lambda_{max} = 259$ mμ). Degradation of the polymer to the mononucleotides using 0·3 N-KOH gave an ϵ_P^{260} of 12,400 for the neutralized digest.

(b) *Physical studies on rAU copolymer*

(i) *Sedimentation velocity studies*

Variation of the mean sedimentation coefficient with ionic strength is shown in Table 3. No significant dependence of S on polymer concentration was observed in the concentration range tested (less than 5% variation in the range of 65 to 380 mμmoles per ml.). The integral sedimentation distribution of a typical preparation is shown in Fig. 2.

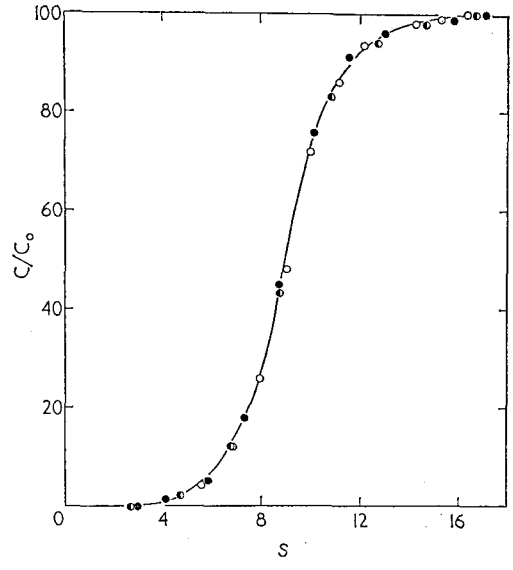

FIG. 2. *Integral sedimentation distribution of rAU copolymer in* 0·05 M-*sodium citrate buffer.* Distribution was calculated from densitometer tracings of u.v. photographs taken 8 (◐), 16 (○) and 24 (●) min after reaching 59,780 rev./min. Nucleic acid concentration was 150 mμmoles/ml.; temperature during the run was 25°C.

(ii) *Viscometry*

Table 3 shows the variation of the reduced viscosity, calculated as η_{sp}/c, as a function of the ionic strength of the solution. For the purposes of this work the concentration dependence of the reduced viscosity was neglected. At the concentrations of rAU used the intrinsic viscosity, obtained by extrapolating the reduced viscosity to zero concentration, differed from the reduced viscosity by less than 10%.

Figure 3 shows the variation of specific viscosity with temperature. The significance of the initial drop in viscosity will be discussed later. As in the case of dAT, the temperature at the midpoint of the rise in viscosity during thermal melting (60·8°C) agrees well with the T_m value (60·5°C) found by optical density melting.

TABLE 3

Influence of ionic strength on the hydrodynamic properties of rAU copolymer

$-\log M_{Na}$	$S_{25,w}$	Reduced viscosity	Calculated molecular weight
	s	dl./g	g/mole
0	8·7	1·64	309,000
1	8·8	1·67	304,000
2	8·5	1·85	299,000
3	7·7	2·43	294,000

Salt concentrations were adjusted using sodium citrate buffer and do not include the salt present as nucleic acid counterion. Sedimentation coefficients were determined at 25°C and corrected to the viscosity of water (Schachman, 1959). Total nucleic acid concentration in sedimentation velocity determinations was approximately 0·15 μmole/ml. Reduced viscosity is specific viscosity divided by concentration in g/dl. and was determined at a concentration of from 2·9 to 3·9 μmoles/ml., at 25°C. Molecular weight was calculated from the Scheraga–Mandelkern equation assuming $\bar{v} = 0{\cdot}50$ and $\beta = 2{\cdot}5 \times 10^6$.

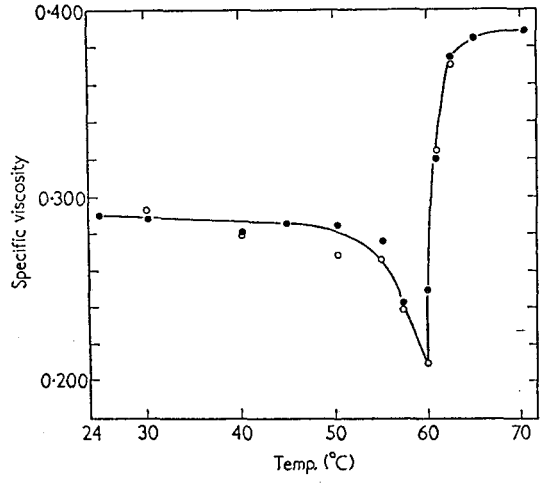

FIG. 3. *Viscometric melting of rAU copolymer.* Data were obtained in 0·05 M-sodium citrate buffer at a nucleic acid concentration of 4·75 μmoles/ml. Full circles show values during heating; open circles show values during cooling.

(iii) *Estimation of molecular weight*

Molecular weight values calculated from the sedimentation and viscosity data using the Scheraga–Mandelkern equation (Scheraga & Mandelkern, 1953) are shown in Table 3. Values of $\beta = 2{\cdot}5 \times 10^6$ and $\bar{v} = 0{\cdot}50$ were assumed (Eigner, 1960; Luborsky & Cantoni, 1962). The size of the product does not bear any simple relationship to the size of the primer. The primer in these studies had an average molecular weight estimated from sedimentation and viscosity measurements of 8×10^6, while the product was approximately 1/25 this size. The tailing of the sedimentation distribution indicates the existence of molecules having an S of at least 16. These data suggest that either the enzyme produces a considerable range of product sizes or that the product is subjected to degradation during synthesis and isolation.

(iv) *Optical density melting*

On heating, solutions of rAU undergo a sharp optical density transition at a temperature characteristic of the salt concentration (Fig. 4). The breadth of the transition at 0·01 M-sodium ion concentration is comparable to that found for the

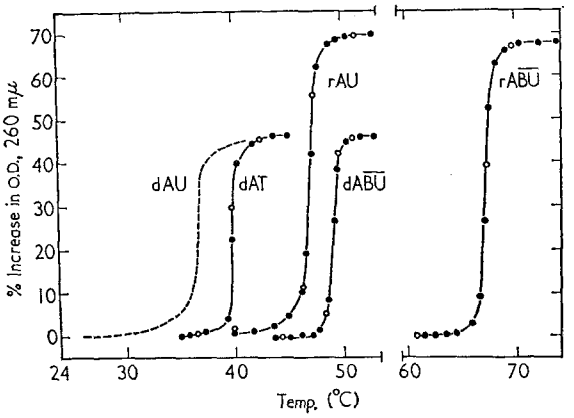

FIG. 4. *Temperature melting of the alternating copolymers in* 0·01 M-*sodium citrate buffer.* Samples were dialysed against a common solution of 0·01 M-sodium citrate buffer. The profile of the curve shown for dAU was obtained on melting in 0·008 M-sodium citrate buffer and has been displaced to the T_m value predicted for 0·01 M-sodium concentration (see Fig. 5). Full circles show values during heating; open circles show values during cooling.

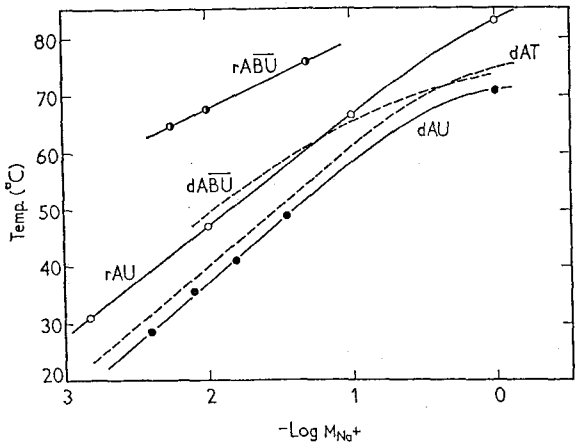

FIG. 5. *Effect of salt concentration on the melting temperature of the alternating copolymers.* The midpoint of the thermal transition T_m is shown as a function of the negative logarithm of the sodium ion concentration. Data shown are for rAB̄U (◑), rAU (○) and dAU (●) in sodium citrate buffers. The upper and lower dashed lines show values obtained by Inman & Baldwin (1962*a*) for dAB̄U and dAT, respectively.

deoxyribocopolymers, but the increase in optical density at 260 mμ (hyperchromicity) observed during melting of rAU and rAB̄U (65 to 70%) is considerably greater than that found with comparable deoxycopolymers (40 to 45%). The variation of the T_m of rAU and dAU with the logarithm of the sodium ion concentration is shown in Fig. 5. It is seen that the thermal stability of the rAU helix is greater than that of

the dAT helix over the entire range of salt concentrations tested, and greater than that of dA$\overline{\text{BU}}$ only above 0·056 M-sodium ion concentration. The deoxyuridine analogue, dAU, has an even lower T_m than dAT, indicating that the enhanced thermal stability of rAU is not due to the substitution of uracil for thymine. As in the case of the deoxyribocopolymers, substitution of $\overline{\text{BU}}$ for U results in a greatly enhanced thermal stability. In each case the difference in T_m between the $\overline{\text{BU}}$- and U-containing polymers diminishes as the ionic strength is increased. Melting curves for rAU preparations handled as described under Methods were reversible; the original optical density was regained even on rapid cooling.†

(v) *Optical rotation measurements*

Optical rotatory dispersion measurements for rAU in the helix form were done in 0·10 M-NaCl, those for the coil form in water under conditions such that the T_m of the polymer was below 30°C (Table 4). Due to the uncertainty in the low angles actually measured, the values of [α] for the rAU coil must be considered only as approximations to the actual values.

TABLE 4

Optical rotatory dispersion of rAU copolymer in the helix and coil forms

λ	[α]	
	Coil	Helix
mμ		
365·4	+18°	+1025°
404·7	− 8°	+ 746°
435·8	−41°	+ 592°
546·1	−22°	+ 342°
588·9	−	(+ 286°)

Optical rotation measurements were made at a concentration of 6·1 μmoles/ml. The uncertainty of the determinations was estimated to be ±7% with a minimum error of ± 25°. The value for $[\alpha]_D$ was obtained by extrapolation along a plot of $1/[\alpha]$ against λ^2 (Yang & Doty, 1957). For further details see Text.

(c) *Attempted physical formation of an RNA : DNA hybrid*

The success of Inman & Baldwin (1962*b*) in forming a hybrid helix of dAT and dA$\overline{\text{BU}}$ by physical means prompted an attempt to make a dAT : rAU hybrid. With dAT and dA$\overline{\text{BU}}$, formation of hybrid occurs only under certain conditions.

† Preparations of rAU which had not been dialysed against high levels of EDTA showed striking changes in hydrodynamic properties and in temperature melting in high and low salt, which were consistent with an appreciable content of bound polyvalent metal ions. The viscosity observed for such preparations in 1 M-salt was time-dependent, increasing rapidly. The appearance of a precipitate in the viscometer indicated that this time dependence was probably due to aggregation. Optical density melting of such preparations in 10^{-3} M-Na^+ showed a broadened transition with melting occurring over a 10°C range. The melting profile was no longer symmetrical, but became increasingly gradual at the higher temperatures. In 1 M-Na^+ there was the sharp transition characteristic of EDTA-dialysed preparations, but the curve was not reversible; the optical density transition on cooling was broadened, and the final optical density after cooling was from 10 to 15% higher than before melting, indicating that considerable degradation of the rAU had occurred. Degradation of RNA by metal ions at high temperatures has been reported previously (Matsushita & Ibuki, 1960; Milligan & Berg, unpublished results).

(1) The Na^+ concentration must be such that the T_m values are the same for both parent helices.

(2) The concentration of each of the parent helices must be at least 5 μmoles/ml.

(3) The mixture must be cooled slowly from a point above the thermal transition of the two helices.

The dAT : dA$\overline{BU}$ hybrid was found to have a buoyant density in cesium sulfate intermediate between those of the parent helices. Since dAT, rAU and dA$\overline{BU}$ are separable from one another in a cesium sulfate density gradient this technique was used to assay for hybrid formation. However, since dAT and rAU do not have the same T_m at any salt concentration, it was necessary to use dA$\overline{BU}$ as the DNA component. dA$\overline{BU}$ and rAU each melt at about 63°C at 0·056 M-sodium ion concentration.

A solution of dA$\overline{BU}$ and rAU containing 8·5 μmoles/ml. of each in 0·056 M-sodium citrate was cooled slowly from 70° to 30°C over 48 hours and a sample then subjected to density gradient centrifugation. Bands of density intermediate between dA$\overline{BU}$ and rAU were not seen under conditions where roughly 10% conversion of the parent helices to hybrid would have been detected.

(d) *Are hybrid molecules formed during RNA synthesis?*

In spite of the failure to demonstrate the physical formation of a dA$\overline{BU}$: rAU hybrid, we examined the possibility that such a hybrid might be formed during the enzymic reaction. dAT was used to prime the synthesis of an approximately equal amount of rAU and the preparation was deproteinized and dialysed to remove triphosphates. The melting profile of the mixture is shown in Fig. 6. No suggestion

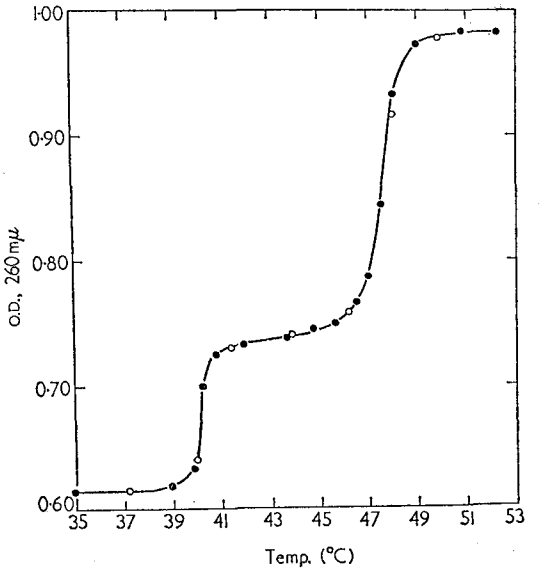

FIG. 6. *Temperature melting of a dAT-primed reaction product in* 0·01 M-*sodium citrate buffer.* An equal synthesis mixture as described in Fig. 7 was deproteinized by treatment with phenol and passage through XE-64 as described in Materials and Methods, then dialysed against 4 changes of 0·5 M-sodium citrate buffer for 48 hr, then 2 changes of 0·01 M-sodium citrate buffer for 24 hr. A portion was then subjected to temperature melting. Full circles are values during heating; open circles are values during cooling.

of a hybrid with an optical density transition between dAT and rAU is apparent. When such a mixture is subjected to density gradient centrifugation in cesium sulfate (Fig. 7) two sharp bands are seen corresponding to the rAU product and the dAT primer; no bands of intermediate density are seen.†

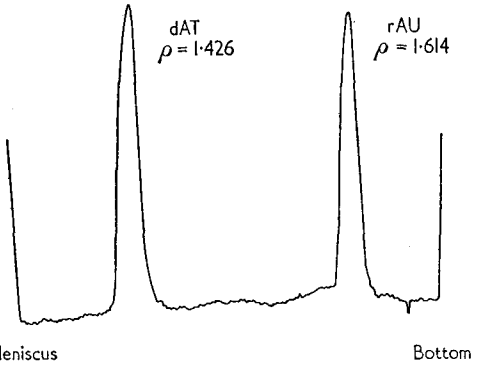

FIG. 7. *Cesium sulfate density gradient centrifugation of a dAT-primed reaction product.* The Figure is a densitometer tracing of a u.v. photograph taken after 12 hr centrifugation at 59,780 rev./min at 25°C. The cell contained 0·45 ml. of 40·8% by weight cesium sulfate and 30 mμmoles of total nucleic acid. The nucleic acids came from a dAT-primed synthesis of rAU in which the reaction was stopped (by addition of sodium citrate buffer to 1·0 M) when the rAU formed was equal to the dAT primer added. The reaction mixture was then dialysed for 24 hr against 2 changes of 1 M-sodium citrate buffer, containing 0·01 M-EDTA, and a sample used for density gradient examination.

4. Discussion

(a) *Structure of the rAU copolymer*

The physical properties of the rAU copolymer strongly suggest that this molecule is a rigid, rod-like helix at temperatures below the helix–coil transition. The major lines of evidence supporting this structure are as follows.

(1) There is a sharp optical density transition with increasing temperature. In 0·01 M-salt the width of the melting transition is from 3 to 4 degrees, comparable to values found for dAT and for natural DNA, and unlike the value of 40 to 50 degrees usually found with natural RNA preparations. Together with the known sequence of rAU, this indicates an alternating base pairing in the rAU helix.

(2) The viscosity and sedimentation coefficient for rAU show little dependence on the ionic strength of the solution. There is only a 1·5-fold variation in viscosity of rAU solutions on going from 1 M-salt to 0·001 M-salt. Such minor changes are also found with dAT (Inman & Baldwin, 1962*a*) and are probably due to secondary salt

† All RNA preparations that we have examined having sedimentation coefficients greater than about 4 S appear to band in a cesium sulfate gradient in an aggregated or precipitated form. (A precipitated band is most easily detected by allowing the rotor to come to rest after a run, removing it gently onto its side, and shining a light through the cell. Under these conditions little disturbance of the gradient is found and a precipitated band is seen as a cloudy layer in the cell.) This aggregation led to difficulties which somewhat limited the utility of the technique in these cases. Buoyant densities observed with different preparations of rAU varied somewhat and seemed to depend in part on the ionic history of the preparation. Polyvalent metal ions increased the aggregation and rAU preparations which had not been dialysed against EDTA frequently gave multiple bands. Further, the band width observed gives no information as to the size of the RNA preparation being examined.

effects (Eisenberg, 1957). Data for ribosomal RNA, which is thought to exist in a coil form, show a 50-fold variation in viscosity over a similar range of salt concentration (Cox & Littauer, 1962).

(3) The values of the intrinsic viscosity and sedimentation coefficient for rAU are characteristic of the rod-like DNA molecule, and differ appreciably from those of natural RNA. Table 5 summarizes the values of the sedimentation coefficient and

TABLE 5

Summary of hydrodynamic properties for different nucleic acid preparations

Nucleic acid	$S_{20,w}$	Intrinsic viscosity	Molecular weight
	s	dl/g	g/mole
rAU copolymer	7·8	1·89	318,000
Calf thymus DNA, sonic fragments†	6·9	1·77	300,000
Ribosomal RNA‡	14·5	0·272	460,000

† Doty, McGill & Rice (1958); ‡ Kurland (1960).

Data for rAU copolymer were determined as in Table 3, but in 0·05 M-sodium citrate. The sedimentation coefficient was corrected to water at 20°C; the intrinsic viscosity was obtained by extrapolation of the reduced viscosity to zero polymer concentration and was also corrected to 20°C. Data for calf thymus DNA and ribosomal RNA are taken from the references noted and were determined in 0·1 M-salt.

viscosity which are found for three nucleic acid preparations of approximately equal molecular weight. The thymus DNA fragments prepared by exposure to sonic vibrations are assumed to have the usual DNA helical structure (Doty, McGill & Rice, 1958), while the ribosomal RNA behaves as a flexible polyelectrolyte (Kurland, 1960). There is good correspondence for these hydrodynamic parameters between rAU and DNA, but not with ribosomal RNA.

(4) Preliminary studies by D. R. Davies§ indicate that the X-ray pattern of the rAU copolymer is that of a helical polymer. The pattern more closely resembles that of helical RNA (Spencer, Fuller, Wilkins & Brown, 1962) than that of the *B* form of DNA. Although highly crystalline, the molecules in the fibers from our present preparations are not sufficiently oriented to allow further conclusions.

(5) Electron micrographs of rAU preparations taken by R. James and M. Beer‖ were consistent with the picture of the rAU copolymer as a rod of diameter approximately 20 to 30 Å and a mean length of somewhat less than 0·5 μ. The specimens prepared for electron microscopy show a tendency to aggregate, and this precludes any definite conclusion about the existence of branching in the rAU molecule.

§ Fibers of the sodium salt of rAU were drawn and their X-ray patterns studied by Dr. Davies of the National Institutes of Health who kindly communicated these conclusions.

‖ Electron microscope examination of rAU samples prepared by shadowing and uranyl or thallous ion staining was generously carried out by Mr. Richard James and Dr. Michael Beer of the Johns Hopkins University.

(b) *Comparison of the properties of rAU with those of similar polymers*

Comparison of the properties of rAU with those of dAU and dAT is of special interest since it is the first instance in which RNA and DNA helices of identical sequence have been compared. In general two sorts of changes might be expected to result from the substitution of ribose for deoxyribose in a nucleotide. Changes may occur due directly to the addition of the 2′-hydroxyl group. Thus, an additional center of asymmetry could account for the much higher optical rotation at the RNA helix compared to the DNA helix. Secondly, changes in the physical structure of the helix due to the ribose substitution might be manifested in changes in the properties of the helix such as its thermal stability. Differences between dAT and rAU could also result from the substitution of U for T.

The optical density melting behavior of rAU is generally quite similar to that of dAU and dAT. There is the same sharp increase in optical density at a characteristic melting temperature and the melting observed is completely reversible, even on quick cooling. The graph of $-\log(Na^+)$ against T_m (Fig. 5) for rAU is parallel to that of dAU and dAT at moderate ionic strengths, indicating a similar influence of the salt concentration on the stability of the helix. There are, however, quantitative differences between the two. In spite of its much lower molecular weight, the rAU shows a T_m which is higher than that of dAT and dAU at all salt concentrations tested. Thus, although the substitution of uracil for thymine lowers the thermal stability of the helix slightly (Shugar & Szer, 1962), the substitution of ribose for deoxyribose leads to an appreciable increase in stability. The unexpected increase in T_m for rA$\overline{\text{BU}}$ over rAU, 20°C, as compared to the 12°C spread between dA$\overline{\text{BU}}$ and dAU indicates that the quantitative effects of substitution can differ considerably between the RNA and DNA helices. A similar striking effect of substitution on the RNA helix was noted by Shugar & Szer (1962) in comparing the thermal stabilities of the rA : rT and rA : rU helices. It is of interest that the T_m values reported (Steiner & Beers, 1961) for the homopolymer RNA helix rA : rU are considerably lower than those found here for rAU at equivalent salt concentrations. The spectra of rAU preparations above and below the melting temperature (Fig. 1) show that on forming the helix there is a shift in λ_{max} from 259 to 261 mμ, and the appearance of a distinct shoulder at 280 mμ. Both effects seem to be characteristic of helix formation from polynucleotides containing adenine and uracil or thymine, and are also found with dAU, dAT (Gellert, 1961) and with rA : rU (Steiner & Beers, 1961). The origin of the shoulder at 280 mμ is under study (Gellert, 1961).

The breadth of the melting transition for dAT increases considerably as the ionic strength of the solution increases (Inman & Baldwin, 1962*a*). This effect is smaller for rAU; thus at 0·001, 0·01 and 0·10 M-salt concentrations, 90% of the optical density change had occurred over a range of 2·1, 3·8 and 5·4°C, respectively, while the corresponding figures found for dAT were 0·5, 2·2 and 4·5°C.† In the case of dAT, it was suggested (Inman & Baldwin, 1962*a*) that this might reflect "folding" at temperatures below the optical density transition. This difference in behavior may be diminished in the RNA helix for some structural reason or it may be due to the shorter chain length of the rAU.

When the helix–coil transition of rAU is followed viscometrically one does observe changes prior to the optical density transition, as in the case of dAT and dA$\overline{\text{BU}}$

† The ordinate in Fig. 4 of Inman & Baldwin (1962*a*), which shows the width of dAT melting curves *versus* $\sqrt{(\text{M-Na}^+)}$, should read 0 to 20°C not 0 to 2·0°C.

(Inman & Baldwin, 1962*a*). When the viscosity of an rAU solution is measured at the ambient temperature there is a sharp decrease in the viscosity prior to the actual transition, followed by a sharp increase in the optical density melting zone. This indicates that there is probably a collapse of the helix prior to melting. For rAU, however, the onset of the viscosity decrease comes at a temperature which is much closer to the optical density melting zone than for dAT and the drop in viscosity is much smaller than for dAT. Again, this suggests that this folding is much reduced in rAU. It is important to note that, unlike dAT, the viscosity melting curve is reversible. The reasons for this are not entirely clear. One might speculate that the reversible melting of rAU reflects the melting and reformation of a hairpin-like helix formed from a single strand, while the (time-dependent) irreversibility in the case of dAT reflects the transition of a helix composed of two separate strands into two separate hairpin-like helices. The increase in viscosity found on melting dAT and rAU, which is anomalous by comparison with natural DNA, has been discussed by Inman & Baldwin (1962*a*).

A notable difference between the ribo- and deoxyribo-alternating copolymers is found in the hyperchromic effect obtained on denaturing the helix. The increase is 40 to 45% for the deoxyribo-copolymers and natural DNA, but it is approximately 65 to 70% for the ribo-copolymers. The latter value is considerably greater than the 55% increase found by Doty, Boedtker, Fresco, Hall & Haselkorn (1959) for the melting of the rA : rU polymer, another helical RNA. It seems possible, however, that the value found for rA : rU is low since, on melting, the rA forms an ordered structure which is hypochromic with respect to the true random coil (Fresco & Klemperer, 1959). A hyperchromic effect of 65 to 70% may prove to be characteristic of a completely helical RNA.

The two nucleic acid helices also differ in optical rotation. The rAU helix shows a specific rotation approximately twice that of dAT at all wavelengths tested (Barrand, unpublished observations). This may also be a general property of the RNA helix. Values of $[\alpha]_D$ for rA : rU have been reported ranging from +240° to +300° (Steiner & Beers, 1961; Doty *et al.*, 1959), quite close to the value of +290° estimated for rAU. On denaturation, the specific rotation of rAU solutions falls to approximately zero, although a precise value for the specific rotation of the coil is not available.

(c) *The use of rAU as a model RNA helix*

Doty *et al.* (1959) suggested the use of model compounds such as rA : rU for estimation of the helical content of natural RNA; their estimates ranged from 40 to 60% for different natural RNA preparations. Recently it has been suggested that amino acid-acceptor RNA may contain as much as 90% helix (Spencer *et al.*, 1962). If one chooses rAU as a model RNA helix, and uses the values obtained for total hyperchromic effect and optical rotation to estimate the helical content of such acceptor RNA in solution at 25°C in 0·1 M-salt, the values obtained by Doty are slightly decreased. On the basis of the data of Tissières (1959) and Ofengand, Dieckmann & Berg (1961), one would predict that less than 40% of the acceptor RNA nucleotides are in helical regions.

(d) *Attempted formation of DNA : RNA hybrids with rAU*

The separate melting of dAT and rAU in mixtures of the two, and the failure to obtain a dA$\overline{\text{BU}}$: rAU hybrid under conditions known to lead to hybrid formation

between dAT and dA$\overline{BU}$ suggest that the hybrid is less stable than either of the parent helices. Thus it fails to form under the conditions used, the parent helices reforming preferentially. The failure to isolate a hybrid in the enzymic reaction can be explained if the DNA helix remains intact during copying (Zubay, 1962) or if a hybrid is formed, but dismutation into free RNA and DNA occurs either during copying or later during isolation. The latter possibility cannot apply to natural DNA since when single-stranded DNA is used as primer for the enzyme, a hybrid molecule is formed which is stable under the conditions used above (Chamberlin & Berg, 1963). Studies of the RNA polymerase reaction using natural double-stranded DNA primers presently support a conservative form of copying (Hurwitz *et al.*, 1962; Geiduschek *et al.*, 1961). Such a process might occur if a hybrid were formed during copying, but the DNA : RNA (hybrid) bonds were less stable than the DNA : DNA hydrogen bonds. The second (free) strand of the DNA helix would then continuously displace the newly synthesized RNA strand from the priming DNA strand with the reformation of the original double-stranded DNA structure (Paigen, 1962).

We should like to acknowledge here the contributions of Dr. Peter Dunlop who participated in the early stages of this investigation, and who first noted the large hyperchromic effect accompanying the thermal denaturation of rAU. We are especially indebted to Mr. Eliot Elson, who allowed us to include his unpublished data on the spectral properties and melting behavior of dAU copolymer.

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